

United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 117

Supplemental Assay Method for Titration of Pseudorabies
Virus Neutralizing Antibody
(Constant Virus - Varying Serum Method)

Date: June 6, 2005

Number: VIRSAM0117.02

Supersedes: MVSAM0117.01, Dated August 3, 1998

Standard Requirement: 9 CFR 113.213

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* serum neutralization (SN) test which utilizes cytopathic effects (CPE) or fluorescent antibody (FA) technique in a cell culture system to determine the SN antibody titer against pseudorabies virus (PRV). The SN assay uses a constant amount of virus to test varying dilutions of serum. The assay meets the requirements in the Code of Federal Regulations, Title 9 (9 CFR) to test serum samples collected from vaccinated and control swine for potency testing of inactivated PRV vaccines.

2. Materials

2.1 Equipment/Instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 36°± 2°C, 5 ± 1% CO₂, high humidity incubator (Model 3158, Forma Scientific Inc.)

2.1.2 2 Water baths

2.1.3 Inverted light microscope (Model CK, Olympus America Inc.)

2.1.4 Fluorescent microscope (Model BH2, Olympus America Inc.)

2.1.5 96-well cell culture plates

2.1.6 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

2.1.7 Micropipettors: 200-µl and 1000-µl single channel; 300-µl x 12-channel

2.1.8 12 x 75-mm polystyrene tubes

2.2 Reagents and Supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

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2.2.1 PRV reference virus, Shope strain [available from the Center for Veterinary Biologics (CVB)]

2.2.2 Madin-Darby bovine kidney (MDBK) or other permissive cells free of extraneous agents as tested by 9 CFR

2.2.3 Minimum Essential Medium (MEM) (Media 20030)

2.2.3.1 9.61 g MEM with Earles salts without bicarbonate

2.2.3.2 1.1 g sodium bicarbonate

2.2.3.3 Q.S. to 1000 ml with deionized water, adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl)

2.2.3.4 Aseptically add 5 ml lactalbumin hydrolysate

2.2.3.5 Sterilize through 0.22- μ m filter.

2.2.3.6 Aseptically add:

1. 10 ml L-glutamine (200 mM)
2. 50 μ g/ml gentamicin sulfate

2.2.3.7 Store at 2°- 7°C.

2.2.4 Growth Medium

2.2.4.1 900 ml of MEM

2.2.4.2 Aseptically add 100 ml gamma-irradiated fetal bovine serum (FBS).

2.2.5 Maintenance Medium

2.2.4.1 98 ml of MEM

2.2.5.2 2 ml of FBS

2.2.6 Swine Anti-Pseudorabies Fluorescein Isothiocyanate Labeled Conjugate [available from CVB]

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2.2.7 0.01 M Phosphate buffered saline (PBS) (Media 30054)

2.2.7.1 1.19 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)⁰

2.2.7.2 0.22 g sodium phosphate, monobasic, monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)

2.2.7.3 8.5 g sodium chloride (NaCl)

2.2.7.4 Q.S. to 1000 ml with distilled water

2.2.7.5 Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl).

2.2.7.6 Sterilize by autoclaving at 15 psi, $121^\circ \pm 2^\circ\text{C}$ for 35 ± 5 minutes.

2.2.7.7 Store at $2^\circ - 7^\circ\text{C}$.

2.2.8 80% Acetone

2.2.8.1 80 ml acetone

2.2.8.2 20 ml distilled water

2.2.8.3 Store at room temperature.

2.2.9 A PRV antibody negative control serum

2.2.10 A PRV antibody positive control serum

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in the immunologic basis of SN assays, cell culture techniques, FA test techniques, and in the principles of aseptic technique.

3.2 Preparation of equipment/instrumentation

3.2.1 Set a water bath at $56^\circ \pm 2^\circ\text{C}$.

3.2.2 Set a water bath at $36^\circ \pm 2^\circ\text{C}$.

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3.3 Preparation of reagents/control procedures

3.3.1 Two days prior to test performance

3.3.1.1 Seed 96-well cell culture plates with MDBK cells, in Growth Medium, at a cell count that will produce a monolayer after 2 days of incubation at $36^{\circ}\pm 2^{\circ}\text{C}$. This becomes the MDBK Plate. Growth Medium is changed if excess acidity of the medium is observed as indicated by a change from red to yellow of Growth Medium or cells are not confluent 2 days after incubation.

3.3.2 On day of test performance

3.3.2.1 Working Virus Preparation. Rapidly thaw a vial of PRV Reference Virus in a $36^{\circ}\pm 2^{\circ}\text{C}$ water bath. Dilute the virus in MEM to contain 50-300 50% tissue culture infective dose (TCID_{50})/25 μl . See **Appendix II** for titration of PRV and determination of the dilution containing the optimal virus dose for the test.

3.3.2.2 Virus Back Titration. Make 3 serial tenfold dilutions of Working Virus.

1. Place 0.9 ml of MEM into 3, 12 x 75-mm polystyrene tubes labeled 10^{-1} to 10^{-3} respectively.
2. Transfer 0.1 ml of Working Virus to the 10^{-1} tube; mix by vortexing. Discard pipette tip.
3. Transfer 0.1 ml from the 10^{-1} tube to the 10^{-2} tube; mix by vortexing. Discard pipette tip.
4. Repeat **3.3.2.2(3)** to the remaining tube, transferring from the 10^{-2} to the 10^{-3} tube.

3.3.2.3 On day of MDBK Plate examination

1. Dilute Swine Anti-pseudorabies Fluorescein Isothiocyanate Labeled Conjugate according to manufacturer's instructions.

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3.4 Preparation of the sample (on day of test performance)

3.4.1 Heat inactivate all Test Serum Samples, negative control serum and the positive control serum in a $56^{\circ}\pm 2^{\circ}\text{C}$ water bath for 30 ± 5 minutes.

3.4.2 Prepare serial twofold dilutions of Test Serum Samples, negative control serum, and positive control serum in a 96-well cell culture plate, which becomes the Transfer Plate (**see Appendix I**). Twofold dilutions are made as follows:

1. Add 150 μl MEM to all wells in Rows B-H.
2. Add 150 μl Test Serum Samples, Positive control serum, and Negative control serum to Rows A and B. Mix Row B with the multi-channel micropipettor (4-5 fills). The same tips may be used throughout.
3. Transfer 150 μl from Row B to Row C. Mix Row C with the multi-channel micropipettor (4-5 fills).
4. Continue as in **3.4.2(3)** for the remaining rows. Discard 150 μl from all wells in Row H.
5. Add 150 μl of Working Stock Virus to all wells of the Dilution Plate. Tap plates gently to mix. Incubate for 60 ± 10 minutes at $36^{\circ}\pm 2^{\circ}\text{C}$ to allow for neutralization of virus. The addition of virus is considered to be an additional twofold dilution of Test Serum Samples.

4. Performance of the test

4.1 Decant Growth Medium from a MDBK Plate.

4.2 Inoculate 50 μl /well of each Virus-Test Serum or Virus-Control Serum mixture into 5 wells/dilution of a MDBK Plate.

4.3 Inoculate 25 μl of each dilution of Virus Back Titration into 5 wells of a MDBK Plate. Add an additional 25 μl of MEM to all wells of the Back Titration.

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4.4 Maintain 2 or more wells on a MDBK Plate as uninoculated cell controls.

4.5 Incubate MDBK Plates for 60 ± 10 minutes at $36^{\circ} \pm 2^{\circ}\text{C}$.

4.6 Add 200 μl /well of Maintenance Medium to all wells (do not remove virus serum mix). Incubate MDBK Plate for 4 days \pm 1 day postinoculation at $36^{\circ} \pm 2^{\circ}\text{C}$.

4.7 CPE counting is the primary method of determining the TCID_{50} .

4.7.1 At four days postinoculation, examine the wells with an inverted microscope. The CPE of PRV is visible as grape-like clusters of rounded cells in the cell monolayer where the cells have been destroyed by the virus.

4.7.2 Record the number of wells/dilution showing any characteristic CPE of PRV for each Test Serum Sample and Virus Back Titration.

4.7.3 Calculate the TCID_{50} of the Working Dilution of virus through the Virus Back Titration using the Spearman-Kärber method as modified by Finney.

Example:

10^{-0} dilution of Working Virus = 5 of 5 wells CPE Positive
 10^{-1} dilution of Working Virus = 5 of 5 wells CPE Positive
 10^{-2} dilution of Working Virus = 3 of 5 wells CPE Positive
 10^{-3} dilution of Working Virus = 0 of 5 wells CPE Positive

Titer = $(x - d/2 + [d \cdot s])$ where:

x = Log_{10} of lowest dilution (=0)
d = Log_{10} of dilution factor (=1)
s = Sum of proportion of CPE Positive

$$\frac{5}{5} + \frac{5}{5} + \frac{3}{5} + \frac{0}{5} = \frac{13}{5} = 2.6$$

Titer = $(0 - 1/2 + [1 \cdot 2.6]) = 2.1$
Antilog of 2.1 = 125.9

Titer of the Working PRV is 125 TCID_{50} /25 μl dose in the test.

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4.7.4 Calculate the endpoint for each Test Serum, Positive Control serum and Negative Control Serum using the Spearman-Kärber method. The endpoints of these sera are reported as SN titer which corresponds to the reciprocal of the highest serum dilution that neutralizes the reference PRV.

Example:

1:2 dilution of Test Serum = 5 of 5 wells CPE Negative
1:4 dilution of Test Serum = 5 of 5 wells CPE Negative
1:8 dilution of Test Serum = 3 of 5 wells CPE Negative
1:16 dilution of Test Serum = 0 of 5 wells CPE Negative

Titer = $(x - d/2 + [d \cdot S])$ where:

x = \log_{10} of lowest dilution (=0.3)
d = \log_{10} of dilution factor (=0.3)
S = Sum of proportion of CPE negative

$$\frac{5}{5} + \frac{5}{5} + \frac{3}{5} + \frac{0}{5} = \frac{13}{5} = 2.6$$

Titer = $(0.3 - 0.3/2 + [0.3 \cdot 2.6]) = 0.93$
Antilog of 0.93 = 8.5

Titer of the Test Serum = 9

4.8 If CPE is difficult to interpret, an FA Test may be conducted as follows:

4.8.1 Decant media from the MDBK Plate.

4.8.2 Fill wells with 80% acetone.

4.8.3 Incubate at room temperature for 15 ± 5 minutes.

4.8.4 Decant the 80% acetone from the MDBK Plate and air dry at room temperature.

4.8.5 Pipette 60 ± 10 μ l of Swine Anti-Pseudorabies Fluorescein Isothiocyanate Labeled Conjugate into all wells. Incubate for 45 ± 15 minutes at room temperature.

4.8.6 Rinse by filling the wells completely in PBS allow to stand for 5 ± 2 minutes, and decant.

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4.8.7 Repeat for a total of 2 washes, decant, and allow to air dry or dry at $36^{\circ}\pm 2^{\circ}\text{C}$.

4.8.8 Examine wells with a fluorescent microscope.

4.8.9 A well is considered positive if typical nuclear, apple-green fluorescence is observed.

4.8.10 Record and calculate as in **4.7.4**.

5. Interpretation of the test results

5.1 The test is invalid if visible contamination or serum toxicity is observed in wells of any dilution of a Test Serum Sample.

5.2 The test is invalid if CPE or fluorescence is observed in any of the control wells.

5.3 For a valid assay, the Virus Back Titration must have between 50-300 TCID₅₀/25 μl .

5.4 The Positive Control serum titer must be within the value of the mean titer \pm one dilution.

5.5 The Negative Control serum must be negative at the 1:2 dilution

6. Report of test results

Record all test results on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.213, U.S. Government Printing Office, Washington, DC, 2004.

7.2 Banks M, Cartwright S. Comparison and evaluation of four serological tests for detection of antibodies to Aujeszky's disease virus. *Vet Rec* (1983) 113:38-41.

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7.3 Conrath TB.: Handbook of Microtiter Procedures. 1972. Clinical and Research Applications Laboratory, Cooke Engineering Company, Alexandria, VA.

7.4 Finney DJ. Statistical Method in Biological Assay. 3rd ed. 1978. Charles Griffin and Company, London, pp. 394-401.

7.5 Geval SH, Joo HS, Mourning JR, McPherson SW, Goval K. Comparison of three serotests for the detection of pseudorabies antibodies in pigs. *Comp Immunol Microbiol Infect Dis* (1987) 10(3-4):67-71.

7.6 Oren SL, Swenson SL, Kinker DR, Hill HT, et al. Evaluation of serological pseudorabies tests for the detection of antibodies during early infections. *J Vet Diag Invest* (1993) 5(4):529-33.

7.7 Parker RA, MA Pallansch. Using the virus challenge dose in the analysis of virus neutralization assays. *Statistics in Medicine*, (1992) 11:1253-1262.

7.8 Rose NR, Friedman H, Fahey JL, eds. *Manual of Clinical Laboratory Immunology*. Chapter 11: Neutralization Assays. 1986. ASM, Washington, D.C.

7.9 Weir DM, Ed. *Handbook of Experimental Immunology*. Volume 3. Application of Immunological Methods. Blackwell Scientific Publications, Edinburgh, England. Chapter 40: Immunological Methods in Virology.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **2.2.3.2** The formulation of MEM was altered to adjust the concentration of sodium bicarbonate to 1.1 grams/liter from 2.2 grams. Penicillin, streptomycin, and amphotericin B were removed from the formulation.
- **2.2.7** Phosphate buffered saline (PBS) has been added.

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- **2.2.9** PRV antibody negative control serum has been added.
- **2.2.10** PRV antibody positive control serum has been added.
- **3.3.2.1 Appendix II** has been added.
- **3.4.1/3.4.2** Positive and Negative Control serums have been added.
- **4.3** 50 µl dilution of Virus Back Titration has been changed to 25 µl. The addition of 25 µl of MEM has also been added.
- **4.7.3** Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.
- **5.4/5.5** Added Positive and Negative Control titer interpretation guidelines.
- **7.1** Added reference to the Code of Federal Regulations (9CFR).
- The refrigeration temperatures have been changed from 4° ± 2°C to 2°- 7°C. This reflects the parameters established and monitored by the Rees system.

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9. Appendices

Appendix I

Transfer Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS1	TS1	TS1	TS1	TS1	TS1	CC	CC	TS2	TS2	TS2	TS2
B												
C												
D												
E												
F												
G												
H												

TS= Test Serum
CC= Cell Control

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Appendix II

Titration of PRV and determination of the dilution containing the optimal virus dose for the Beta SN test

1. Label eight 17 x 100-mm plastic tubes by identifying one tube as 10^{-1} , one tube for 10^{-2} , etc. through 10^{-8} . The number of tubes required will depend on the endpoint of the reference virus
2. Dispense 4.5 ml of cell culture medium in each of the tubes
3. Rapidly thaw, using a 36°C water bath, one vial of Reference cytopathic BVD virus, and using a pipette or pipettor and tip, transfer 0.5 ml of virus to the first tube (10^{-1})
4. Discard pipette or pipettor tip, and vortex the 10^{-1} virus dilution tube
5. Using a new pipette or pipettor tip, transfer 0.5 ml of the 10^{-1} dilution of virus to the 10^{-2} tube, discard pipette or pipettor tip, and vortex the 10^{-2} dilution. Continue diluting in a similar fashion through the last tube, using a new pipette or pipettor tip for each transfer.
6. Transfer 0.025 ml of each virus dilution to each of five wells of a MDBK cell plate as prepared in **3.3.1.1**. The plate is then incubated in a $36^{\circ}\pm 2^{\circ}\text{C}$, 5% CO_2 incubator for 4-5 days.
7. The titration plate is read for CPE, and the results are recorded as number of wells exhibiting CPE per number of wells inoculated with each virus dilution.
8. Using the Spearman-Kärber method, calculate the endpoint of the PRV virus titration. The titer is expressed as \log_{10} tissue culture infective doses fifty (TCID_{50}) per 25 μl dose.

Example:

10^{-1} dilution PRV = 5 of 5 wells CPE Positive
 10^{-2} dilution PRV = 5 of 5 wells CPE Positive
 10^{-3} dilution PRV = 5 of 5 wells CPE Positive
 10^{-4} dilution PRV = 5 of 5 wells CPE Positive
 10^{-5} dilution PRV = 3 of 5 wells CPE Positive
 10^{-6} dilution PRV = 0 of 5 wells CPE Positive

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Titer = $(X - d/2 + [d \cdot S])$ where:

Titer = $(1 - 1/2 + [1 \cdot 4.6]) = 5.1$

The antilog of 5.1 = 125893

Therefore, a 1:125,893 dilution of the virus should yield a virus concentration of 1 TCID₅₀ per 25 µl. Assuming a 200 TCID₅₀ per 25 µl is the optimal dosage of virus to use in the SN test, divide the 125,893 by 200 to obtain 629.46. The dilution factor to obtain 200 TCID₅₀ per 25 µl would then be 1:629. This Working Reference PRV dilution must be confirmed by performing a back titration. A series of three to five dilutions of virus bracketing the expected dilution may be tested with back titrations of each. Adjustments from the original calculated dilution may need to be made to obtain the correct working virus dose.